Evaluation of chemicals for toxic & teratogenic effects using the chick embryo as the test system-FDA Contract #71-331-FDA Compound #71-17-**Sterculia Karaya**-No Date

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FDA CONTRACT 71-331

EVALUATION OF CHEMICALS FOR TOXIC AND TERATOGENIC EFFECTS USING THE CHICK EMBRYO AS THE TEST SYSTEM

STERCULIA KARAYA: FDA 71-17

WARF INSTITUTE, INC.
MADISON, WISCONSIN

FDA CONTRACT 71-331

EVALUATION OF CHEMICALS FOR TOXIC AND TERATOGENIC EFFECTS USING THE CHICK EMBRYO AS THE TEST SYSTEM

Objective: To determine the toxic and teratogenic effects of GRAS List compounds when injected into the air cell and yolk of fertile chicken eggs.

Procedure:

A. Test System and Incubation Procedures:

Fertile hatching eggs were chosen from a single comb white leghorn breeder flock. The eggs were candled and graded to eliminate internal and external defects; blood and meat spots, tremulous air cells, rough or cracked shells. The eggs chosen for injection weighed from 23 - 26 ounces/dozen and were not washed or dipped. The eggs were gathered within 48 hours of the injection or incubation and were held at 50 - 60°F. and 60 - 80% relative humidity. The breeder ration fed the flock was formulated by the breeder to meet or exceed the recommendations of the Nutrient Requirements of Poultry, Number 1 - 1971, National Academy of Sciences, and contained no additions of antibiotics, arsenicals, nitrofurazones or similar chemical additives. The breeder flock was blood tested and negative for pullorum-typhoid and mycoplasm gallispecticum.

Eggs were incubated in Jamesway 1080 forced air incubators equipped with automatic controls to regulate temperature, humidity and egg turning. Temperature and relative humidity were maintained at 99.5°F. and 86°F.* wet bulb respectively for the first 18 days of incubation and eggs were turned each two hours. The eggs were then transfered to the hatcher in 3½" x 5" x 25" covered hardware cloth hatching baskets for the hatching period. Temperature in

* 86°F. wet bulb refers to temperature of wet bulb apparatus in standard incubator hatching equipment and is equivalent to approximately 56% relative humidity.

the hatcher was maintained at 98.5oF. and relative humidity at 86oF. wet bulb. When the humidity had risen to 88 degrees as a result of moisture generated by hatching the hatcher was adjusted to hold 88oF. wet bulb relative humidity until the chicks were removed on the morning of the 23rd day of incubation.

Prior to incubating or hatching each setting of eggs and following each hatching the incubator and its metal parts were thoroughly cleaned by vacuuming and washing with a 200 ppm solution of "ROCCAL" which contains 10% alkyl (C12, C14, C16 and related alkyl groups from C8 to C18) - dimethyl benzyl ammonium chloride. The sanitized surfaces were allowed to completely air dry prior to the introduction of eggs. Following each transfer of eggs to the hatching compartment, and when temperature and humidity had returned to normal levels, the hatcher and the eggs it contained were fumigated by combining 10 grams of potassium permanganate crystals and 20 grams of 37% formaldahyde solution.

B. Test Sample Preparation and Administration:

The test sample was taken up in an appropriate solvent to facilitate administration at the levels chosen. Sterile glassware, syringes and needles were employed to prepare and administer the test sample or solvent. Eggs previously selected were candled and the location of the air cell marked with pencil. The eggs were then randomized into the experimental groups. Injection of solvent or test sample dilution was accomplished by placing the material on the air cell membrane or by injection into the yolk sac. These administrations were made at both 0 and 96 hours of incubation. For the 0 hour experiments the eggs were assumed to be fertile; however, in the 96 hour experiments the eggs were candled as previously described and only those eggs with a well developed 96 hour embryo were selected for use.

1. Air Cell Administration:

The eggs were wiped at the injection site with 70% ethanol and allowed to air dry. A hole measuring approximately 6mm was then drilled over the air cell in each egg using a "Dremel Moto-tool", model 270. The cutter employed deflected the shell fragments upwards and outwards. Remaining shell membrane fragments were removed with a small

forceps and the surface of the egg membrane visually examined for damage. The solvent or test sample was then deposited on the egg membrane with a model SB2 Syringe Microburet. Immediately following, the hole was sealed with ½" Scotch Brand transparent tape. Two additional groups of eggs were normally included with each air cell experiment; a group which had been drilled, shell membrane fragments removed, and sealed only and a group of control eggs which had received no treatments whatsoever.

2. Yolk Administration:

The eggs were placed within a Fisher Scientific "Isolator/Lab" equipped with plastic irises through which the hands and forearms were placed during injection. Prior to injection the eggs and miscellaneous required equipment were submitted to a fumigation of 1.8 grams of potassium permanganate crystals and 3.6 grams of 37% formaldahyde. The eggs were held in this atmosphere for 30 minutes prior to further handling.

Each egg was then wiped at the injection site with 70% ethanol and allowed to air dry. A small hole was engraved directly over the air cell with a Burgess model V-13 Vibro-Graver. Care was taken not to damage the membrane attached to the shell. The surface of the egg at the engraved site was vacuumed to remove the shell particles produced. The egg was then slid onto the needle of the Syringe Microburet with the egg horizontal on its long axis until the top of the egg reached the hub of the 1" - 25 ga. hypodermic needle. Following the injection of the material into the yolk sac, the egg was carefully withdrawn from the needle and the hole sealed with transparent tape. The hypodermic needle was carefully wiped with a sterile gauze pad prior to the next injection. As in the air cell administration, normally two additional groups of eggs were included in each yolk experiment; a group which had been drilled, pierced with the hypodermic needle and sealed only and a group of control eggs which had received no treatment other than fumigation.

Following the air cell and yolk injections the eggs were identified as to experiment and group with a No. 3 lead pencil and were then incubated as described above.

C. Test Profile:

The work was divided into one or more Preliminary Range Finding Experiments, two Dose-Response and Teratogenic Experiments, and Ancillary Investigations (Post Hatch Trials).

1. Preliminary Range Finding Experiments:

The objective of these trials was to locate the approximate LD-50 of the test sample. This data was used to design the dose levels for the Dose-Response trials. The test sample and solvent were administered by two routes; air cell and yolk, and at 0 and 96 hours of incubation. In general, at each route and time of incubation, 5 volumes of test sample dilution were administered together with 5 levels of solvent at the same volumes. Control eggs were also usually included as described above. Normally 10-20 eggs were used per group in these trials. When necessary these trials were repeated in an effort to locate the approximate LD-50 for the test compound.

Beginning on the 6th day of the incubation, the eggs set in the Preliminary Range-Finding Experiments were candled daily and non-viable embryos removed. These embryos were examined grossly for determination of developmental age and evidence of teratogenic effect, however, mortality was the main parameter in these trials. The remaining eggs were transferred to the hatching compartment on the 18th day of incubation and allowed to hatch. The resultant chicks and non-viable embryos were examined grossly for teratogenic effects and all pertinent data was recorded. An estimate of the LD-50 for the test compound was then made.

2. Dose-Response and Teratogenic Experiments:

Based upon information from the Preliminary Range-Finding Experiments, the Dose-Response Experiment was designed, employing 5 levels of sample dilution expected to produce mortality from the background level up through approximately 90%. Five volume levels of solvent were included as solvent controls at each route and time of administration. Normally 10 eggs/group were used in the solvent series with 50 eggs/group for the test sample dilutions. Twenty eggs/group were normally included for the drilled or pierced and non-treated controls. Two such experiments were conducted for each sample so that ultimately 100 eggs were tested on each test dilution at each route and stage of incubation.

The eggs set in these experiments were candled daily beginning on the 6th day of incubation and the non-viable embryos removed for examination as previously described. Where necessary, embryos were examined with the aid of a dissecting microscope. Remaining embryos were transferred to the hatching compartment on the 18th day of incubation and allowed to hatch. The apparently normal chicks were then removed from the hatching trays and examined externally for anomalies.

Remaining non-viable embryos and chicks which were alive but unable to hatch were individually examined externally for abnormalities. These non-viable embryos, chicks which were alive but unable to hatch, and a portion of the normal chicks were examined in one aspect by X-ray. The chicks and embryos which had been X-rayed and all remaining normal chicks were then examined internally for possible anomalies of the viscera. All pertinent data were recorded.

3. Post Hatch Trials:

Apparently normal chicks were chosen from one 50 egg experiment for this portion of the study.

Generally 20 chicks (straight-run) were wing banded from each level chosen and were placed in Jamesway electrically heated battery brooders. Central Soya Chick Starter was fed as the sole ration to 8 weeks of age and Central Soya Grower from 8 weeks of age to termination. These diets were non-medicated. The chicks chosen were usually from the approximate LD-50 and no-effect levels for the test compound from each route of administration and time of incubation. Negative control, untreated chicks, were also included. In some cases chicks were chosen from groups where a relatively high incidence of anomalies were seen rather than from the LD-50 or no-effect levels specifically. Body weight data were collected weekly through 4 weeks of age and bi-weekly to termination. Average group feed consumption was recorded periodically.

4. Histopathology:

A random sampling of birds from selected groups were specified for histologic examination. These chicks comprised 5 males and 5 females from the test groups selected and 5 males and 5 females from a negative control group. Groups to be sampled were selected on the basis of observations of specific effects and a judgment made as to what groups would give the most information from the limited histopathologic examination.

The chicks sacrificed were either day old or varying ages in a Post Hatch Trial. The following tissues were collected, trimmed, dehydrated, embedded in paraffin, sectioned and stained with hematoxilin and eosin:

1. Thyroid
2. Liver
3. Spleen
4. Pancreas
5. Lung
6. Heart
7. Kidney
8. Gonad
9. Bursa

The prepared slides were examined and remarkable alterations noted.

Results:

The data developed in the testing of Sterculia Karaya are presented in the following tables:

Sterculia Karaya

Table 1 - Albumen At 0 Hours
Table 2 - Albumen At 96 Hours
Table 3 - Yolk At 0 Hours
Table 4 - Yolk At 96 Hours
Table 5 - Histopathology - Day Old Chicks

In Tables 1 through 4, the following comments apply:

Column 1 gives the dose administered milligrams per kilogram, respectively. (The milligrams per kilogram figure is based on an average egg weight of fifty grams).

Column 2 is the total number of eggs treated.

Column 3 is the percent mortality, i.e., total non-viable divided by total treated eggs.

Column 4 is the total number of abnormal birds expressed as a percentage of the total eggs treated. This includes all abnormalities observed and also toxic response such as

edema, hemorrhage, hypopigmentation of the down and other disorders such as feather abnormalities, significant growth retardation, cachexia, ataxia or other nerve disorders.

Column 5 is the total number of birds having a structural abnormality of the head, viscera, limb or body skeleton expressed as percentage of the total eggs treated. Toxic response and disorders such as those noted for column 4 are not included.

Column 2 through 5 have been corrected for accidental deaths if any occurred. Included in these columns are comparable data for the solvent-treated eggs and the untreated controls.

The mortality data in column 3 have been examined for a linear relationship between the probit percent mortality versus the logarithm of the dose. The results are indicated at the bottom of each table.

The data of columns 3, 4 and 5 have been analyzed using the Chi Square test for significant differences from the solvent background. Each dose level is compared to the solvent value and levels of percent mortality or percent abnormalities that are significant (probability of being the same is 5% or less) are indicated by an asterisk in the tables. All values so indicated have a higher incidence than the solvent values.

Discussion:

The comments and data which follow concern the results obtained when Sterculia Karaya was employed in the test system.

A. 0.12N HCL solution was chosen as the solvent for Sterculia Karaya. The tendency of the sample to cause gelling in solution prevented the use of distilled water as a solvent. The 0.12N HCL reduced the gelling somewhat allowing a greater use concentration. The inability of the test solution to move through the air cell membrane necessitated the substitution of the albumen injection to replace the air cell treatments. In this procedure, the egg was prepared and the injection delivered as in a yolk treatment except that the needle used was only of sufficient length to reach into the albumen.

Average mortality in solvent treated eggs was approximately 16-18% in 0 and 96 hour treatments and 96 hour yolk. At 0 hour yolk, the average solvent mortality was 42%. Significantly increased levels (P .05) of mortality were seen in test sample treated groups at each time and route. The mortality did not appear to be dose related. LD-50 values were calculable at 0 hour albumen, 0 hour yolk and 96 hour yolk. The LD-50 values obtained are of questionable value since they are projected responses to dose levels which were

not administered in these experiments.

Percent total abnormal birds was not significantly elevated in 0 hour albumen treatments as compared to the solvent control. Anomalies seen were of low incidence and generally had been seen in control eggs for this time and route or in the flock background at similar levels. The flock background is an accumulation of observations on all the drilled, pierced and untreated control eggs carried in 50 egg experiments using flock N_1 eggs. A total of 3,315 eggs were involved.

Percent total abnormal birds was significantly elevated in 96 hour albumen treatments at 3.5, 7.0 and 70.0 mg/kg. Dwarfism, retarded development was the principal contributor to the total and averaged approximately 13% at these dose levels. This condition was seen at a 4% level in solvent eggs for this time and route and at 3.4% in the flock background. Seen in the approximately 500 test sample treated eggs tested in 50 egg experiments for this time and route were additional anomalies at the frequencies indicated; clubbed down (10), bulla on abdomen (1), crossed beak (1), short mandible (1), long mandible (1), anophthalmia (2), cleft palate (1), exencephaly (1), acrania (1), celosomia (1), small kidney (2), missing kidney (1) and light down (6). None of these additional anomalies were seen in the control eggs for this time and route, however, with the exception of long mandible and anophthalmia, had been seen previously in the flock background.

In the 0 hour yolk treatments, percent total abnormal birds was elevated only at 56.0 mg/kg where dwarfism, retarded development, was seen in 13% of embryos examined. This was the only anomaly encountered at this dose level. Seen in the remaining approximately 450 eggs treated with the test sample at this time and route were crossed beak (1), short maxilla (1), cataract (1), anophthalmia (2), excencephaly (2), kyphosis (1), enlarged heart (1), cyst on heart (1), enlarged kidney (1), celosomia (2) and leg micromelia (1). None of these anomalies were seen in the control eggs for this time and route. In addition; short maxilla, cataract, anophthalmia, kyphosis, enlarged heart, cyst on heart and leg micromelia had not been seen in the flock background.

Percent total abnormal birds was significantly elevated at 96 hour yolk only in a 10 egg experiment at 42.0 mg/kg. Retarded development was the only anomaly seen at this dose level. A very low incidence of other anomalies was seen in the remaining eggs dose at this time and route and for the most part had been previously seen in the flock background. An exception was a single 19 day embryo at 7.0 mg/kg with bilateral anophthalmia,brain hypoplasia, bulla on skull, acrania and maxilla agenesis. Of these anomalies, only acrania had been seen previously in the flock background.

Dwarfism, retarded development, was a frequent observation in this experiment. To clarify our designation of dwarfism we will explain our classification procedure. At the first candling, day 5 or 6, we did not classify any embryos as retarded unless they were

alive and definitely younger in development than 5 or 6 days. In subsequent candling any dead embryo judged by size to be 3 days behind in development was labeled as slight dwarfism, 4 days behind was labeled moderate dwarfism and 5 days or more behind was labeled severe dwarfism. If embryos were removed alive, 1 day behind was labeled slight, 2 days behind was labeled moderate and 3 days behind was labeled severe dwarfism. At hatch time an 18 day embryo was classified slightly dwarfed, a 17 day embryo was classified as moderate dwarfism and a 16 day embryo was classified as severe dwarfism. One might suspect that at the toxic levels administered, embryo development could be delayed due to metabolic or nutritional alterations that produced temporary growth depression which would not result in a permanent growth defect. Chicks which hatched were of normal size and no evidence of permanent growth retardation was observed.

Hatched chicks were not carried in a post hatch grow-out trial. Day old male and female chicks were selected from 96 hour yolk groups receiving 3.5 and 56.0 mg/kg of the test sample and from the solvent control for sacrifice and collection of tissues. The tissues of chicks receiving the test sample were compared histologically with those of the solvent control. The alterations seen were minimal in nature.

X-ray examinations did not reveal abnormalities not already seen during gross examination.

Conclusion:

Under the conditions specified for this trial, Sterculia Karaya produced minimal mortality above that caused by the 0.12N HCL solvent. At times and routes where percent total abnormal birds was significantly increased, dwarfism (retarded development) was the primary contributor to the anomalies seen. Since chicks which hatched was of normal size and development, this retarded development was considered a termporary toxic response of the embryo to the solvent and/or test sample. Most other anomalies seen were of low incidence and for the most part had been encountered in the flock background. However, very low incidence of serious anomalies of the head, limbs and spine was seen which had not been seen in the flock background or control eggs and because of their serious nature, may suggest that further investigation with this compound is indicated.

Signed R W Bodden

By and For WARF Institute, Inc.

November 27, 1974

Test Sample: Sterculia Karaya

Identification: FDA 71-17

Solvent System: .12N Hydrochloric Acid

Breeder Flock: N-1

Preliminary Range Finding Experiments

Experiment No.	Initiated
32	6/12/72
49	9/25/72

Dose Response Experiments

Experiment No.	Initiated
52	10/23/72
56	11/27/72

Table 1

Sterculia Karaya
Albumen At 0 Hours

Dose Mg/Kg	Number Of Eggs	Percent** Mortality	Percent Abnormal	
			Total	Structural
70.0	99	23.23	5.05	1.01
42.0	10	10.00	10.00	.00
35.0	98	19.38	12.24	3.06
21.0	10	10.00	.00	.00
17.5	100	28.00*	10.00	1.00
10.5	10	30.00	.00	.00
7.0	110	13.63	4.54	.00
3.5	110	11.81	6.36	3.63
.12N Hydrochloric Acid	150	16.00	6.00	2.00
Pierced Control	119	24.36	11.76	1.68
Control/ Control	260	11.53	4.61	1.92

** LD-50 8,567 mg/kg

* Significantly different from solvent ($P \leq .05$)

Table 2

Sterculia Karaya
Albumen At 96 Hours

Dose Mg/Kg	Number Of Eggs	Percent** Mortality	Percent Abnormal	
			Total	Structural
70.0	98	31.63*	23.46*	3.06
42.0	10	30.00	.00	.00
35.0	100	29.00	14.00	5.00
21.0	10	10.00	10.00	.00
17.5	100	24.00	11.00	1.00
10.5	10	10.00	.00	.00
7.0	110	30.90*	12.72	.00
3.5	110	36.36*	15.45*	1.81
.12N Hydrochloric Acid	148	18.24	6.75	.67
Pierced Control	120	15.83	3.33	.83
Control/ Control	260	11.53	4.61	1.92

** Slope is negative, LD-50 not achieved

* Significantly different from solvent ($P \leq .05$)

Table 3

Sterculia Karaya
Yolk At 0 Hours

Dose Mg/Kg	Number Of Eggs	Percent** Mortality	Percent Abnormal	
			Total	Structural
56.0	50	46.00	26.00*	.00
42.0	10	90.00*	30.00	.00
28.0	99	43.43	19.19	7.07
21.0	10	70.00	.00	.00
14.0	100	37.00	16.00	.00
10.5	10	80.00*	10.00	.00
7.0	100	44.00	12.00	2.00
3.5	109	39.44	15.59	3.66
1.4	60	43.33	11.66	.00
.12N Hydrochloric Acid	149	42.28	10.73	2.01
Pierced Control	119	24.36	11.76	1.68
Control/ Control	260	11.53	4.61	1.92

** LD-50 245,468 mg/kg

*Significantly different from solvent ($P \leq .05$)

Table 4

Sterculia Karaya
Yolk At 96 Hours

Dose Mg/Kg	Number Of Eggs	Percent** Mortality	Percent Abnormal	
			Total	Structural
70.0	50	28.00	6.00	2.00
56.0	100	24.00	14.00	1.00
42.0	10	70.00*	40.00*	.00
28.0	100	21.00	8.00	1.00
21.0	10	30.00	20.00	.00
14.0	100	21.00	9.00	1.00
10.5	10	30.00	10.00	.00
7.0	100	27.00	12.00	1.00
3.5	60	11.66	5.00	.00
1.4	10	10.00	.00	.00
.12N Hydrochloric Acid	149	16.77	10.73	2.68
Pierced Control	120	15.83	3.33	.83
Control/ Control	260	11.53	4.61	1.92

** LD-50 147,792 mg/kg

* Significantly different from solvent ($P \leq .05$)

Table 5

Sterculia Karaya
Histopathology Day-Old Chicks

Histologic Observations	.12N HCL (10 Birds)	96/Y 56 MG/KG (10 Birds)	96/Y 3.5 MG/KG (10 Birds)
Thyroid			
less collid but normal		2	6
diffuse mild congestion		1	
area of congestion surrounding gland			1
Liver			
diffuse mild/moderate pigmentation	6	1	2
diffuse mild cloudy swelling	6		
diffuse mild/moderate vacuolization	3		
fatty change mild	2		
Kidneys			
atrophic glomeruli	3		